Clin. exp. Immunol. (1991) **84**, 213–218 ADONIS 000991049100131Z

Immune responsiveness and parasite-specific antibody levels in human hepatobiliary disease associated with *Opisthorchis viverrini* infection

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(Accepted for publication 1 December 1990)

SUMMARY

Opisthorchis viverrini infection is associated with human hepatobiliary disease and cholangiocarcinoma, but the role of the immune response in the pathogenesis of infection is unclear. Here ultrasonography was used to examine the biliary tracts of residents from an endemic community. Delayed-type hypersensitivity responses to unrelated antigens, and fluke-specific IgG and IgA levels in serum of this group were also examined. Relationships between immunological parameters, intensity of infection and radiologically measured variables are reported. Immune responsiveness to unrelated antigens did not vary with intensity of parasite infection or disease status. Of all the variables, IgG levels were most markedly elevated in disease cases compared with normal subjects and were closely associated with gall bladder size and dysfunction. This is consistent with the hypothesis that an immunopathologic mechanism is involved in opisthorchiasis and suggests that antibody levels may be useful in screening populations for fluke-associated hepatobiliary disease.

Keywords parasite-specific antibodies gall bladder *Opisthorchis viverrini* ultrasonography ELISA

INTRODUCTION

The liver fluke, *Opisthorchis viverrini* is a major public health problem in North East Thailand where it is estimated 7 million people are infected (Preuksaraj, 1984). Humans acquire this parasite by eating raw fish containing infective metacercariae. The flukes reside in the small bile ducts and gall bladder and do not undergo systemic migration. Eggs exit the biliary tract and are excreted in faeces.

The most serious manifestation of liver fluke infection is an increased susceptibility to cholangiocarcinoma (CHCA), adenocarcinoma of the biliary tract (Hou, 1956; Flavell, 1981; Harinasuta, Riganti & Bunnag, 1984; Kim, 1984; Elkins *et al.*, 1990). CHCA is the leading cancer in North East Thailand, but it is rare in areas where the fluke is absent (Vatanasapt *et al.*, 1990a, b). Several less severe diseases, e.g. cholelithiasis, cholecystitis and cholangitis, are also associated with *Opisthorchis* (Harinasuta *et al.*, 1984; Dhiensiri *et al.*, 1984; Elkins *et al.*, 1990); these are risk factors of CHCA (Fraumeni & Kantor, 1982; Nagorney & McPherson, 1988).

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The role of the liver fluke in the aetiology of biliary disease and carcinoma remains uncertain. During infection, desquamation of the bile duct epithelium can occur, leading to over-reactive repair, including hyperplasia, fibrosis and goblet cell metaplasia (Hou, 1956; Bhamarapravati, Thamavit & Vajrasthira, 1978; Flavell, 1981; Harinasuta *et al.*, 1984; Kim, 1984). The fluke may release toxins which cause direct damage (Hou, 1956; Kim, 1984; Harinasuta *et al.*, 1984), and/or the changes may be immunopathological (Bhamapravati *et al.*, 1978; Flavell, 1981). Published reports describe mainly damage to the biliary epithelium; the pathogenesis of gall bladder disease and its role in carcinogenesis is unknown.

Wongratanacheewin *et al.* (1987) have shown that *Opisthorchis* infection causes a generalized suppression of immunological responses in hamsters. The survival of malignant cells may be enhanced by parasite-induced impairment of immunosurveillance mechanisms. However, it is not known whether infection suppresses immunity in humans.

Our recent studies demonstrated a high frequency of ultrasound-diagnosed hepatobiliary disease associated with heavy *Opisthorchis* infection in a sample group drawn from a small Northeast Thai village (Elkins *et al.*, 1990). Here we demonstrate close relationships between radiological and immunological data from this group.

Table 1. Summary of the radiological findings for the village residents selected to undergo ultrasonography

Diagnosis	*n*	Mean age (years)	Worm load	IgG*
Normal, no radiological abnormalities	41	40	10·0 (2·8)	0·33 (0·04)
Mild gall bladder disease, thickened wall or enlarged gall bladder, good contraction	15	49	117·3 (56)	0·44 (0·04)
Cholelithiasis	6	51	303·4 (199)	0·59 (0·13)
Chronic cholecystitis, thickened wall, enlarged or contracted organ, poor contraction	12	51	251·5 (80·2)	0·71 (0·09)
Cholangiocarcinoma, suspected if hydrops gall bladder, a mass, dilated common and/or intrahepatic bile ducts	8	50	NA	0·93 (0·09)
Parenchymal disease, increased echoes in the liver	11	49	165 (128)	0·60 (0·12)

* Expressed as the mean (s.e.m.).
NA, Praziquantel contraindicated. The mean egg output of this group was the highest of all groups (Elkins *et al.*, 1990).

MATERIALS AND METHODS

Study design and ultrasonography

The procedures used in the field program and details of the sample group are described elsewhere (Elkins *et al.*, 1990). The project was carried out in Bahn Huai Matho, Changwat Kalasin, North East Thailand, beginning in March 1989. All adults over 19 years of age (total 87) with negative ($n=19$) or high ($>10\,000$ eggs/g; $n=22$) *Opisthorchis* egg counts or with clinical indications (palpable liver, history of jaundice) underwent ultrasonography. The purpose of the study was explained and informed consent obtained from the participants. Subjects fasted at least 6 h before examination using a Toshiba Sonolayer LS-SAL-55AS with a 3·5 mHz linear array probe. Radiological diagnoses and the number of cases observed are given in Table 1. Gall bladder size was defined as its greatest length observed from all planes of view (Everson *et al.*, 1980). Contraction was assessed by remeasuring the organ 30 min after consumption of a fatty meal (Dhiensiri *et al.*, 1984; Hederstrom *et al.*, 1988). Portal vein radicle echoes (PVRE) were scored according to the prominence of echoes along the portal triad (Homeida *et al.*, 1988). All examinations were performed by one experienced radiologist who was unaware of the clinical, parasitological or immunological status of the subjects. Suspected cases of CHCA were investigated further using endoscopic retrograde cholangiopancreatography and computed tomography.

Parasitology

Egg counts were performed using a quantitative formalin ether technique. Worm burdens were determined by recovery of flukes from stools passed during 48 h after treatment with praziquantel (40 mg/kg body weight) and magnesium sulphate purgative (Radomyos, Bunnag & Harinasuta, 1984; Ramsey *et al.*, 1989; Elkins *et al.*, 1991).

Skin tests

The cell-mediated immune responsiveness of 44 of the subjects (all consenting CHCA cases plus randomly selected normal and gall bladder disease cases) was assessed by measuring the induration diameters 48 h after application of seven common antigens and glycerin alone (negative control) to the forearm (Multitest CMI; Institut Merieux). A total skin test index was calculated as the sum of induration diameters to all antigens whose diameters exceeded 2 mm.

Laboratory investigations

Venous blood was collected from each participant, and serum prepared by centrifugation was frozen at −20°C until required. Relative levels of parasite-specific antibodies among subjects were quantified by ELISA as previously described (Wongratanacheewin *et al.*, 1988; Elkins *et al.*, 1991). For the determination of IgG levels, Immulon 2 microtitre plates (Dynatech) were coated overnight with 0·625 μg/ml of somatic antigen extract of adult *Opisthorchis* and test sera were diluted 1/800. To estimate IgA levels, 5 μg of the same antigen was used, and sera were diluted 1/200. After incubation and washing of test sera, peroxidase-conjugated anti-human IgG and IgA (Dakopatts, Glostrup, Denmark) was added for 1 h, then OPD substrate (Sigma) incubated for 30 min. Absorbance at 490 nm was determined by spectrophotometry. The mean absorbance of the negative control serum pool drawn from Thai laboratory staff was 0·17 for IgG and 0·38 for IgA.

Statistical analysis

Analysis of variance (ANOVA) and χ^2 tests were used to test the significance of variation in means (e.g. immunoglobulin levels, egg counts) or frequencies (e.g. increased PVRE) between the disease groups. Correlations between quantitative variables were determined by calculating Pearson's correlation coefficient after data transformation to achieve normality (assessed by Kolmogorov–Smirnov test, $P<0·05$). Multiple regression was used for multivariate examination. SPSS/PC+ software was used for all analyses.

RESULTS

As can be seen from Table 1, many cases of hepatobiliary disease, including suspected early cholangiocarcinoma, were observed by ultrasonography. For further details of these findings and their relationship to the intensity of liver fluke infection, see Elkins *et al.*, (1990). Because of its association with other biliary and renal diseases, parenchymal disease is not included in the statistical analyses, and data are shown separately.

Cell-mediated immune response, fluke infection and biliary disease

Cutaneous reactivity to the seven skin-test antigens followed a normal distribution in the sample group ($n=44$). The propor-

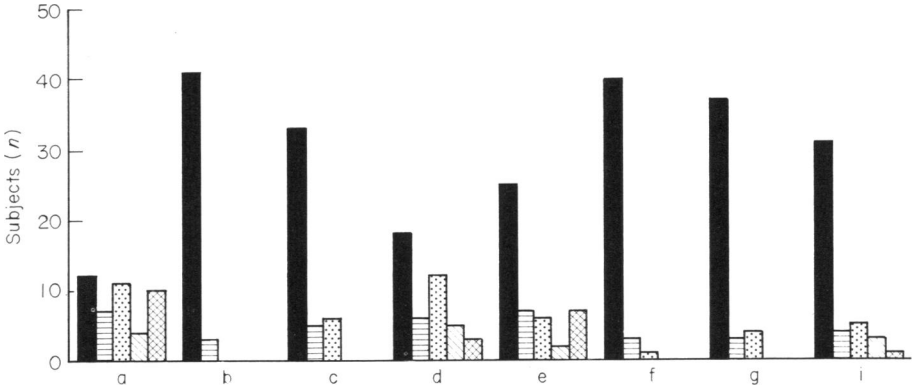

Fig. 1. The distribution of sizes of indurations from individual antigens measured on subjects' forearms 48 h after application of a seven antigen delayed type hypersensitivity test (multi-test CMI). The antigens were: a, tuberculin; b, glycerin (negative control); c, streptococcus; d, candida; e, diptheria; f, trichomonas; g, tetanus toxoid; and i, *Proteus mirabilis*. Induration diameter: ■, <2 mm; ▤, 2–4 mm; ▩, 4–6 mm; ▧, 6–8 mm; ■, >8 mm.

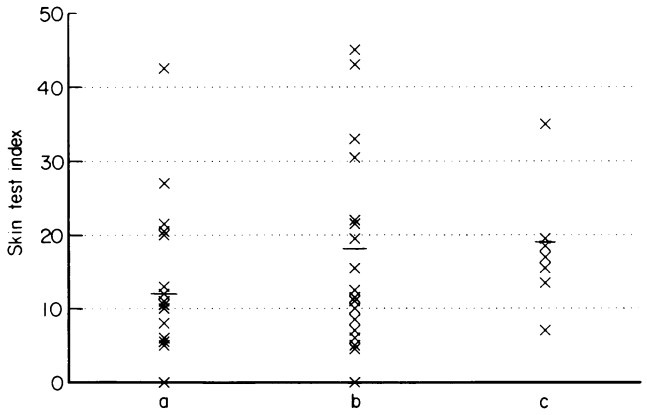

Fig. 2. The skin test indices of individual subjects stratified by ultrasound diagnosis into groups of normal (a); gall bladder disease (b) (mild disease, cholelithiasis and chronic cholecystitis); and suspected CHCA (c). The horizontal bars represent the mean values of the groups.

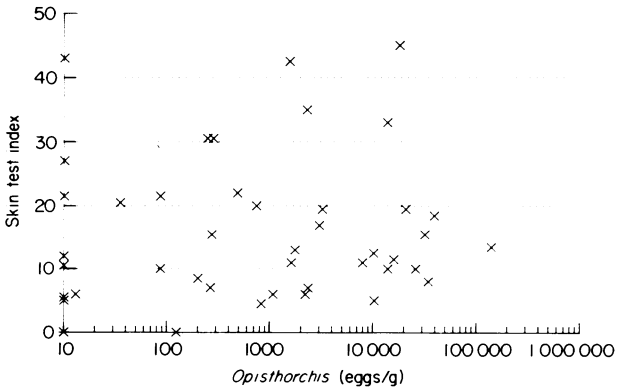

Fig. 3. The skin test indices plotted against the intensity of *Opisthorchis* infection, as estimated by eggs/g of faeces, of 44 individuals in the sample group.

tion of subjects showing a positive reaction varied considerably between antigens (Fig. 1), with tuberculin, candida and diphtheria showing the strongest reactivity. The tuberculin induration size correlated most closely with the total skin test index ($r=0{\cdot}74$). Other antigens, e.g. trichomonas and tetanus toxoid, were rarely recognized and not useful for this group.

Statistical analysis of age, sex, biliary disease (Fig. 2), *Opisthorchis* egg count (Fig. 3), and parasite-specific IgG and IgA showed no significant associations with total skin test reactivity (Pearson's r or one-way ANOVA, $P>0{\cdot}05$). Suspected CHCA cases showed reactivity levels comparable to normal individuals (Fig. 2).

Antibody levels, portal vein radicles, intensity of infection and hepatobiliary disease

In this group, the levels of parasite-specific IgG and IgA correlated significantly with the intensity of fluke worm burden and egg count within individuals ($r=0{\cdot}50$ and $0{\cdot}58$, $P<0{\cdot}001$ for IgG; and $r=0{\cdot}37$ and $0{\cdot}32$, $P<0{\cdot}01$ for IgA) (see Elkins *et al.*, 1990). In addition, highly significant variation in the levels of IgG and IgA was observed between biliary disease groups as diagnosed by ultrasonography (IgG, Fig. 4a, $F=14{\cdot}69$, df=477, $P<0{\cdot}001$; IgA, Fig. 4b, $F=7{\cdot}67$, $P<0{\cdot}0001$). IgG levels were more markedly elevated in disease cases than were *Opisthorchis* egg counts (Fig. 4c, $F=4{\cdot}74$, df=477, $P<0{\cdot}001$) or worm burden ($F=6{\cdot}44$, df=351, $P<0{\cdot}001$) (Elkins *et al.*, 1991). Only 17% of normal individuals had absorbance values for IgG above 0·40, in contrast to 70% of non-malignant disease cases and 100% of CHCA subjects.

Increased PVRE were also frequently observed in association with gall bladder disease and in all cases of CHCA (Table 2a, $\chi^2=48{\cdot}8$, df=4, $P<0{\cdot}001$). Although this radiological parameter is thought to be related to long-standing *Opisthorchis* infection, the associations between PVRE and egg counts, worm burden and age were less striking.

Antibody levels, portal vein radicles and the size and function of the gall bladder

In addition to the overall ultrasound diagnosis, significant associations were observed between IgG levels, PVRE and individual gall bladder abnormalities. Thickened gall bladder wall was observed in 45% of the subjects who also had a

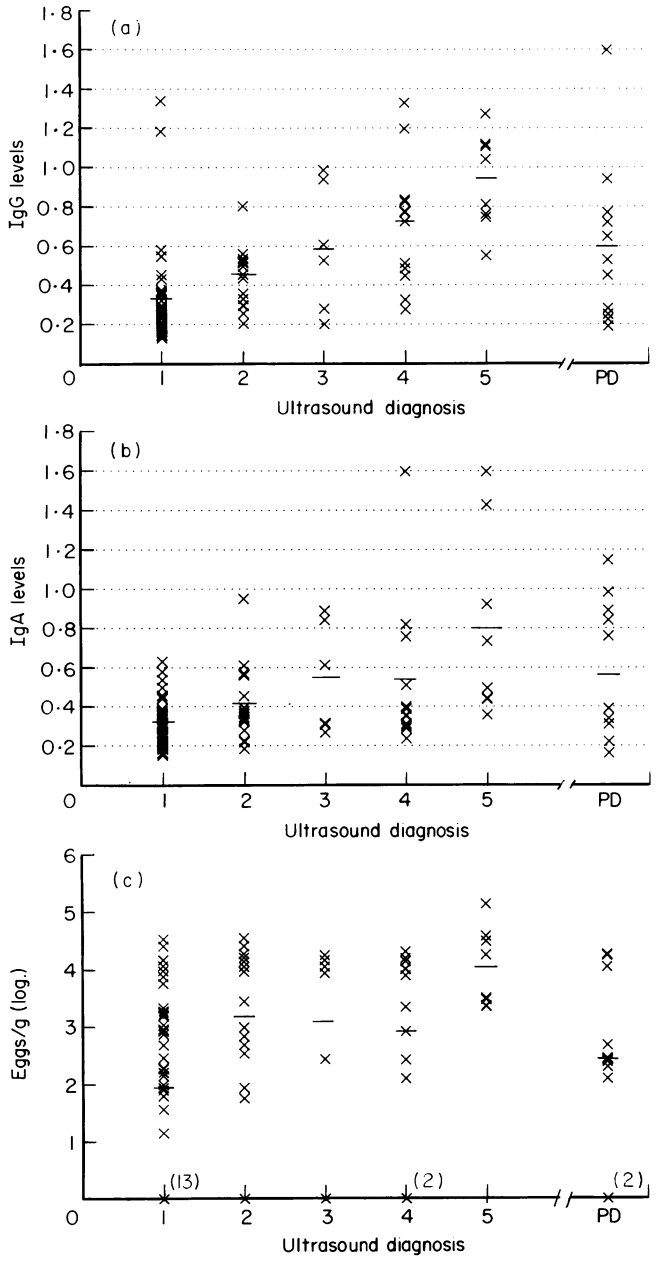

Fig. 4. Parasite-specific antibody levels and intensity of infection in hepatobiliary disease. Absorbance values for IgG (a) and IgA (b) in ELISA and *Opisthorchis* egg counts (c) of individual subjects stratified by ultrasound diagnosis. The diagnoses are coded as: 1, normal; 2, mild gall bladder disease; 3, cholelithiasis; 4, chronic cholecystitis; 5, suspected cholangiocarcinoma; and PD, parenchymal liver disease. The horizontal bars represent the mean values of the groups and s.e.m. for IgG are given in Table 1. Numbers are shown inside (c) to indicate how many cases were negative for fluke eggs, where there were more than 1.

Table 2. Associations between the intensity of portal vein radicle echoes (PVRE) and hepatobiliary abnormalities

	Portal vein radicles		
(a) Relationship with hepatobiliary diagnosis			
Diagnosis	0	1	2
Normal	40	0	1
Mild disease	10	1	4
Cholelithiasis	3	2	1
Chronic cholecystitis	4	4	4
CHCA	0	4	4
Parenchymal disease	3	6	2
(b) Relationship with gall bladder wall thickness			
Gall bladder wall	0	1	2
Normal	43	4	1
Thickened	16	10	13
(c) Relationship with contraction			
Contraction	0	1	2
Normal response	9	1	5
< 50% reduction	6	7	2
No change	0	1	5

PVRE ranked as: 0, normal; 1, mild increase; or 2, moderate to severe increase.

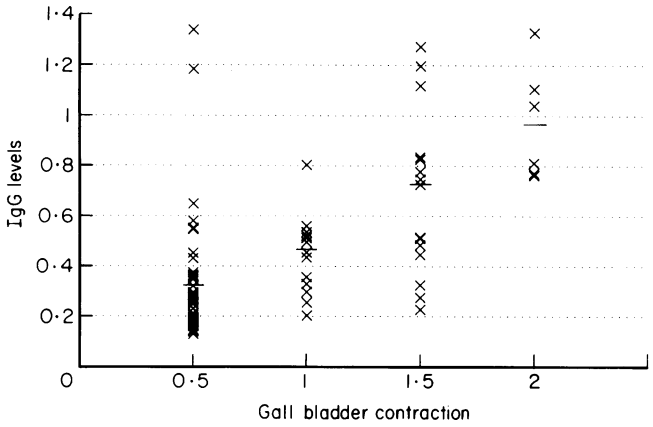

Fig. 5. Parasite-specific IgG levels and gall bladder function. IgG levels of individuals in groups stratified by the degree of gall bladder contraction after fatty meal. Contraction is coded as: 0·5, group not examined because the gall bladder was considered normal (thin walled and not enlarged or contracted) by ultrasonography; 1, good contraction (> 50% reduction in size); 1·5, poor contraction (< 50% contraction); and 2, no contraction (no difference in pre- and post-meal size of the organ). The horizontal bars represents the mean values of the groups.

significantly higher frequency of increased PVRE ($x^2 = 24{\cdot}44$, df = 1, $P < 0{\cdot}001$, Table 2b). Mean IgG levels varied significantly with severity of gall bladder dysfunction (ANOVA, $F = 7{\cdot}3$, df = 233, $P < 0{\cdot}005$, Fig. 5). All subjects with no contraction had increased PVRE, but the frequency of increased echoes differed only slightly between the normal and poor contraction groups ($\chi^2 = 8{\cdot}52$, df = 2, $P < 0{\cdot}025$, Table 2c).

The length of the gall bladder showed similar correlations with egg count, worm burden and IgG levels ($r = 0{\cdot}41$–$0{\cdot}48$). Multiple-regression analysis was performed incorporating egg count and worm burden, age, sex, IgG and IgA levels as independent variables and gall bladder size as the dependent variable. This analysis indicated that IgG level accounted for the

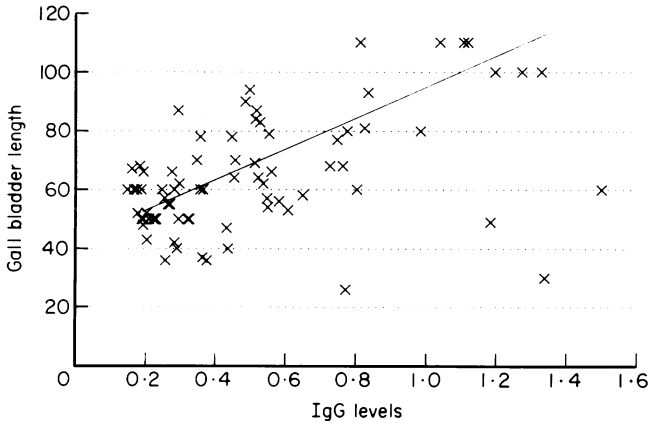

Fig. 6. The relationship between *Opisthorchis*-specific IgG levels and maximum length of the gall bladder as measured radiologically. Linear regression generates the equation $y = 51{\cdot}65x + 43{\cdot}2$, where y = the absorbance value for IgG in ELISA and x = gall bladder length. The regression is highly significant (ANOVA, $F = 81{\cdot}44$, df = 70, $P < 0{\cdot}0001$).

most variability in gall bladder size ($r = 0{\cdot}48$), and inclusion of other variables did not improve the model significantly.

There are four obvious outlying points in Fig. 6; one of which (length, 26 cm) represents a contracted gall bladder, an abnormal state after fasting indicating chronic cholecystitis. Of the three remaining outliers, all had light worm burdens and radiologically normal gall bladders (can also be seen on Figs 4a and 5). Two received anti-helminthic treatment at least twice before this study, which may have influenced both their antibody level and gall bladder status. If these points are excluded, the correlation coefficient between IgG levels and gall bladder length rises from 0·48 to 0·73.

DISCUSSION

Several chronic inflammatory diseases of the gastroenteric mucosa are associated with an increased risk of carcinoma (Fraumeni & Kantor, 1982; Christie, 1987; Petras, Mir-Madjlessi & Farmer, 1987; Nagorney & McPherson, 1988). However, an aetiological agent is established only in a few; this precludes examination of the role of specific responses in pathogenesis. *Schistosoma haematobium* in bladder carcinoma and liver flukes in CHCA provide good models for studying the role of immunopathology in chronic disease and carcinogenesis (Christie, 1987).

A close relationship was observed between parasite-specific antibodies, PVRE and hepatobiliary disease in *Opisthorchis viverrini* infection. More specifically, IgG levels were highly predictive of gall bladder size and contraction, while PVRE were closely linked to gall bladder wall thickness. The intensity of current fluke infection showed weaker associations with these abnormalities.

One possible interpretation of the close relationship reported between IgG levels and the size and function of the gall bladder is that these antibodies have a causative role in the pathophysiology of gall bladder disease. Two mechanisms are proposed. The antibodies may bind host antigens on the gall bladder, eliciting autoimmune damage, as suggested by some investigators for chronic myocardial damage in *Trypanosoma cruzi* (Hudson, 1985). Alternatively, parasite excretory/secretory antigens may adsorb to host cell surfaces leading to antibody binding, complement fixation and chronic inflammation. The proposed immunopathology may involve antigen or isotype-specific IgG, as suggested in filariasis (Hussain, Grogl & Ottesen, 1987), Sowda-type onchocerciasis (Parkhouse & Harrison, 1989) and inflammatory bowel disease (Kett, Rognum & Brandtzaeg, 1987).

The relationship between IgG levels and enlargement and poor function of the gall bladder may be directly causative (as suggested above), but it is equally possible that enlargement of the gall bladder results from other pathological mechanisms and secondarily enhances the leakage of parasite antigens into circulation. This could result in proportionately higher antibody levels, which may or may not participate in the pathological process. Further studies are needed to clarify these possible mechanisms.

Increased echoes along the portal vein radicles were not strongly associated with current intensity of infection or age (duration of infection). However, their increased frequency in disease cases and cases of gall bladder wall thickening supports their association with pathogenesis. Increased PVRE may represent fibrotic change resulting from chronic inflammation, perhaps initiated by specific T cell responses to *Opisthorchis* antigens. Interestingly, thickening of the gall bladder wall and increased PVRE are also observed in Symmers' periportal fibrosis in *S. mansoni* infection (Homeida *et al.*, 1988). Studies are in progress to determine the histopathological equivalent to PVRE in *Opisthorchis* infection and their association with cell-mediated responses.

The lack of a detectable influence of either heavy infection or disease status on cutaneous delayed-type hypersensitivity responses suggests that *Opisthorchis* infection does not induce generalized immunosuppression in humans. These findings, however, should be followed up on larger populations.

The observation that 83% of the normal group and only 27% of the total diseased group had antibody titres less than 0·4 suggests that this assay may be a useful screening tool for the diagnosis of fluke-associated disease. ELISA is quicker and cheaper to perform than the conventional stool examination for fluke eggs. Where the prevalence of infection is high, the presence of eggs may be an incidental finding rather than an indication of involvement in disease. High IgG levels, however, appear to be more predictive of disease than egg counts, except in some recently treated people.

These data are consistent with the hypothesis that biliary tract and gall bladder abnormalities associated with *Opisthorchis* infection, which may create favorable conditions for carcinogenesis, are at least in part immunopathological. IgG may contribute to bile stasis (as risk factor for CHCA) if it plays an active role in the enlargement and malfunction of the gall bladder, while inflammatory responses may elicit chronic cell proliferation and fibrotic damage. Our results do not indicate that parasite-induced immunosuppression is a major factor in tumour development.

ACKNOWLEDGMENTS

This research was generously supported by the Tropical Health Program, Commonwealth Government, Australia. We express appreciation to P. Awacharagan for excellent technical assistance in the ELISA, and to P. Treesarawat, S. Phinlaor, S. Phisaipan, C. Phisaipan, P. Sirisatchang and L. Chaturat for field and laboratory work. The

study would not have been possible without the advice and support of local health personnel, including Dr V. Wechosotsakda, Dr S. Khanthai, S. Ponrueng, B. Singawn and T. Poopahngern. We also thank P. Graves, A. Saul, M. Good, D. McManus and G. Mitchell for helpful comments on the manuscript, D. Battistuta for statistical advice and Dr D. Moss for suggesting the multi-test CMI.

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